



High efficient ethanol and VFAs production from gas fermentation: effect of acetate, gas and inoculum microbial composition

El-Gammal, Maie; Abou-Shanab, Reda; Angelidaki, Irini; Omar, Basma; Sveding, Per Viktor; Karakashev, Dimitar Borisov; Zhang, Yifeng

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7 3 Maie El-Gammal ^a, Reda Abou-Shanab ^b, Irini Angelidaki ^c, Basma Omar ^{a,c}, Per Viktor
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9 4 Sveding ^c, Dimitar Borisov Karakashev ^c, Yifeng Zhang ^{c,*}
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12
13 5 ^a Department of Environmental Sciences, Faculty of Science, Damietta University, 34517
14
15 6 Damietta, Egypt
16

17 7 ^b Department of Environmental Biotechnology, City of Scientific Research and Technology
18
19 8 Applications, Alexandria, 21934, Egypt
20
21

22 9 ^c Department of Environmental Engineering, Building 113, Technical University of Denmark,
23
24 10 DK-2800 Lyngby, Denmark
25
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28 11 *Corresponding author.
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31 12 Dr. Yifeng Zhang
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35 13 Department of Environmental Engineering, Technical University of Denmark, Denmark
36
37

38 14 Tel: (+45) 45251410
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41 15 Fax: (+45) 45933850
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44 16 E-mail address: yifz@env.dtu.dk
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Abstract

In bioindustry, syngas fermentation is a promising technology for biofuel production without the use of plant biomass as sugar-based feedstock. The aim of this study was to identify the optimal conditions for high efficient ethanol and volatile fatty acids (VFAs) production from synthetic gas fermentation. Therefore, the effect of different gases (pure CO, H₂, and a synthetic syngas mixture), media (acetate medium and acetate-free medium), and biocatalyst (pure and mixed culture) were studied. Acetate was the most dominant product independent of inoculum type. The maximum concentration of volatile fatty acids and ethanol was achieved by the pure culture (*Clostridium ragsdalei*). Depending on the headspace gas composition, VFAs concentrations were up to 300% higher after fermentation with *Clostridium ragsdalei* compared to mixed culture. The addition of acetate has a negative impact on the VFAs formation with a varying degree depending on gas compositions.

Keywords: Syngas fermentation; Acetate; Ethanol; *Clostridium ragsdalei*; Gas compositions; Microbial culture

1. Introduction

The extensive consumption of fossil fuel, high energy prices and the need for reducing greenhouse gas emissions have drawn increasing attention to the development of alternative and sustainable energy. Biological fermentation of syngas is a promising emerging technology, expected to play a vital role in achieving this goal. This technology not only produces biofuels and valuable chemicals but also contributes to environmental pollution control. Syngas which essentially consists of a mixture of carbon monoxide, hydrogen, and carbon dioxide derived from the gasification of solid fuels (i.e. coal, petroleum coke, and oil shale), and biomass, or reforming of natural gas, and from manufacturing waste gases, particularly from steel production [1,2]. Syngas platform for biofuels production is a two stage process, where syngas is first produced by gasification and then syngas can be fermented to biofuels. This platform has several advantages over the conventional biochemical methods for biofuel production, such as high specificity of the biocatalyst, and utilization of the whole biomass including lignin [3]. Fermentation of syngas for the production of biofuels can be performed at ambient temperature and pressure and does not require any costly pre-treatment of the feed gas or costly metal catalysts [4].

The most promising bacterial groups for fermenting syngas are acetogens. These unique obligatory anaerobic bacteria have the ability to fix CO₂ using H₂ as an electron donor or CO alone and produce alcohols and organic acids [5]. These microorganisms undergo a set of enzyme catalyzed reactions through an irreversible, non-cyclic pathway known as Wood-Ljungdahl pathway to convert syngas into acetyl-CoA [3]. Acetyl-CoA can subsequently be used for ATP generation needed for microbial biomass building and formation of acetate, ethanol and other byproducts during the later stages of the

pathway [6]. Several mesophilic pure cultures especially within the species *Clostridium* have been used for syngas fermentation [7]. Prominent among these is *Clostridium ragsdalei*, which has been successfully used for syngas fermentation [8,9]. However still important challenges need to be addressed before commercial application. Gas-liquid mass transfer limitations, syngas quality, microbial catalysts and product recovery are the major issues to be addressed in order to make syngas fermentation more economically feasible [10].

Recently mixed culture fermentation has gained more attention due to several advantages compared to the pure culture, such as process robustness during continuous processes and no need for highly sterile conditions [11]. However, systematic comparison of pure and mixed culture syngas fermentation to alcohols and/or acids, which could permit developing efficient biofuels and biochemicals processes, has not been made so far.

In addition to the microbial catalysts, syngas composition in terms of H₂/CO ratio is also an important factor that significantly affects the output of the syngas fermentation process [12]. It has been recently reported that some *Clostridia* species could further reduce the volatile fatty acids (VFAs) to their corresponding alcohols by using syngas as electron donor [13]. Thus, it is of importance to investigate the influence of presence of VFAs (e.g., acetate) on the gas fermentation processes [14].

Based on the points highlighted above, the main objective of this study was to identify the optimal conditions (media, gas compositions and microbial catalyst) for high efficient alcohol and VFAs production from synthetic gas fermentation. Moreover

process performance for VFAs and ethanol production during pure culture and mixed cultures fermentation were compared.

2. Materials and Methods

2.1. Microorganisms

Clostridium ragsdalei P11 (No. 15248, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany) was used as a pure culture for syngas fermentation process in this study. A digested manure (treated cattle manure and wheat straw) was obtained from the effluent of a lab scale anaerobic thermophilic continuous stirred reactor (CSTR) at Technical University of Denmark to use as a source of anaerobic mixed microbial communities for syngas fermentation. The digested manure was pre-treated with heat at 90°C for 15 minutes to inactivate methanogenic archaea and to enrich for anaerobic spore-forming bacteria [15].

2.2. Experimental design

An experimental scheduled (Table 1) was designed to test the effect of three different factors on the fermentation process. The variables tested included two different inocula: pure culture (*C. ragsdalei*) and mixed culture, two media compositions: acetate modified medium (AMM) and acetate free medium (AFM), and three feed gas compositions: Syngas \pm 60% CO \pm 35% H₂ \pm 5% CO₂; 100% CO and 100% H₂ (added only in the acetate modified medium). In the following paper the gas compositions will be referred to as: synthetic syngas (SS), CO and H₂. The response variable for each treatment combination was the concentration of ethanol, acetate and the other VFAs obtained during 10 days of fermentation.

2.3. Inoculum preparation

To reduce the lag phase and ensure adaptation to the syngas, *C. ragsdalei* and mixed culture inocula were propagated under strict anaerobic conditions three times prior to inoculating the batch experiment [16]. Each inoculum was prepared in 250 ml serum bottles with 100 ml of ATCC 1754 medium [2] and 10% (v/v) inoculum in each passage. The bottles were pressurized to 1.5 bar with (60% CO, 5% CO₂ and 35% H₂) syngas mixture by volume and kept at 37°C. The inoculation of a new batch was made when the previous was reaching cell optical density (OD₆₆₀) of about 0.5. The third cultivation was used to inoculate the batch experiment.

2.4. Experimental batch setup

Through this study, the effect of two different media: acetate free medium (AFM) and acetate modified medium (AMM) on the gas fermentation process was studied. The both medium was based on ATCC medium 1754 with the exception that yeast extract and fructose were omitted to minimize the interference of organic compounds during the fermentation [2]. The only difference between the two media was the addition of acetate in (AMM) to a final concentration of 13 mM along with the other chemicals before adjustment of the pH. All the fermentative experiments were carried out in triplicate using 320 mL serum bottles with a working volume of 22 mL. The bottles with the media were sealed with butyl rubber stopper and aluminum cap and then sterilized at 121°C and 15 psi for 20 min. After sterilization, 150 mM MES (2-(N-Morpholino) ethanesulfonic acid sodium salt) buffer solution, vitamin and reducing agents were added using 0.2 µm sterilized filter while flushed with N₂ to keep anaerobic conditions. Adding MES to a concentration of 150 mM and vitamins after autoclaving was inspired by [14]. The detailed composition of trace metals, vitamin and reducing agents stock solutions are presented in Supplementary Information (SI). The initial pH

of the medium was adjusted to 6 ± 0.2 with 1 M KOH and 1 M HCl solutions. Fermentations with either *C. ragsdalei* or the mixed culture were inoculated with 10% (v/v) inocula. All fermentation serum bottles were incubated horizontally (to maximize gas-liquid mixing) at 37°C with a constant agitation of 120 rpm on an orbital shaker for 10 d. Three different gas compositions (Synthetic syngas consisting of 60% CO, 35% H₂, 5% CO₂), 100% CO and 100% H₂ (only added in the acetate modified medium) were injected to the headspace of the fermentation bottles to a final pressure of 1.5 bar. For biotic controls the cultures were kept under 100% N₂ gas phase instead of the other gases.

2.5. Analytical methods

Samples (2 ml) were retrieved from the fermentation broth (to avoid disturbance of the bacteria) on day 4 and 10 for VFA analysis along with optical density and pH measurements. The pH was measured by a digital PHM210 pH meter connected to the Gel pH electrode (pHC3105 - 8; Radiometer analytical). The optical density (OD) was determined using Spectronic 20D+ (Thermoscientific, Soeborg, Denmark). Cell samples were collected in 4 mL cuvettes from the Serum bottles and the OD was measured at 600 nm. After measuring the pH and the optical density, the fermentation samples were prepared as previously described for VFAs and alcohols determination using a gas chromatograph (GC) (Shimadzu GC-2010, Kyoto, Japan), equipped with a flame ionization detector (FID) and a FFAP fused-silica capillary column, 30 m × 0.53 mm I.D., film thickness 1.0 µm, using nitrogen as a carrier gas [17]. The oven temperature was initially set at 50°C for 3.5 min. and then increased 25°C / min to 130°C followed by 10°C /min to 210°C, and kept at final temperature for 10 min. The injection port and detector temperatures were 150°C and 230°C, respectively. Headspace gas samples

were collected using sample lock gas tight syringes every 48 h from the serum bottle reactors and analyzed for CO₂ consumption using a GC (MicroLab, Aarhus, Denmark) with a paralleled column of 1.1 m×3/16 “Molsieve 137 and 0.7 m×1/4” chromosorb 108. Hydrogen concentration was measured by GC-TCD with N₂ as carrier gas. It was fitted with a 4.5m³ mms-m stainless column packed with Molsieve SA (10/80), and the temperature of the injector, detector and oven were 190°C, 110°C and 90°C, respectively. Detection limit for H₂ and CO₂ was 0.06%. All chemicals were purchased from Sigma–Aldrich, unless stated otherwise.

2.6. Statistical analyses and calculation of kinetic parameters

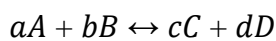
Analysis of variance (ANOVA) and Duncan's new multiple range test (MRT, p < 0.05) were used for multiple comparisons via MSTATC (Michigan State University, East Lansing, MI, USA). Gibbs free energy changes at standard conditions (ΔG°) of possible fermentation reactions by *Clostridium* and mixed cultures are shown in Table 2. According to Coma et al. [18] ΔG is dependent on the concentrations of initial reactants and products through the equation:

$$\Delta G = \Delta G^o + RT \ln(Q) \quad (1)$$

Where R is a constant (0.00829kJ/mol*K), T is the absolute temperature (K) and;

$$Q = \frac{[C]^c[D]^d}{[A]^a[B]^b} \quad (2)$$

Which applies for the general reaction:



3. Results and Discussion

3.1. Growth of pure and mixed culture with synthetic media

The growth profile of *C. ragsdalei* and mixed culture cells in terms of optical density (OD₆₀₀) is shown in Fig. 1. The maximum growth of the *C. ragsdalei* and mixed cultures was observed under the headspace of CO, which was 23% and 84%, respectively, higher than that under SS (Fig. 1). It is more thermodynamically favorable using CO as electron acceptor than other electron donors (e.g., H₂) independent of pH, ionic strength, gas partial pressure, and electron carrier pairs and therefore the bacteria would potentially gain more energy for cell growth by using CO [19,20]. The difference between biotic controls and other samples was most significant for pure culture ($p < 0.05$), where it was clear that fermentation gasses had a positive effect on bacterial growth. Results showed that OD₆₀₀ of the mixed culture under SS and CO was 86.5% and 80%, respectively, significantly lower ($p < 0.05$) compared with the *C. ragsdalei*. Meanwhile there was no significant difference ($p > 0.05$) in OD₆₀₀ between samples with and without acetate.

3.2. Fermentative ethanol and VFAs production

Fig. 2 compares the production profiles of ethanol and VFAs formed by the *C. ragsdalei* under different gas compositions. The metabolites produced during fermentation were ethanol, acetate, propionate, butyrate and valerate. The most dominant product by pure culture fermentation in all gas composition was acetate. The maximum concentration of acetate (17 mmol/l) was observed under a headspace of CO after 10 days. In comparison, the final concentration of acetate under headspaces of SS was 26% lower. Previous studies have also pointed out CO as the preferred reductant over H₂ [19,20]. This could explain the higher formation of acetate under a headspace of CO than the mixture of CO, H₂ and CO₂.

Production of ethanol was observed in both experiments with SS and CO headspace (Fig. 2A and B) with a maximum concentration 1.4 and 1.6 mmol/l after only 4 days of inocubation whereafter the concentration of ethanol decreased. This indicated that ethanol was consumed for the production of higher chained fatty acids through chain elongation. According to Table 2 production of ethanol should be thermodynamically more favorable than acetate. A simultaneous production and consumption of ethanol could therefore explain the relatively low concentrations. From Table 2 (reactions 1- 4) it can be seen that ethanol formation with CO is thermodynamically more favorable than with H₂. This was also supported by Liu et al. [21] stating that most favorable reactions are with low H₂:CO ratios.

However, the current study confirmed that *C. ragsdalei* can grow and construct components needed for growth directly from syngas in yeast free medium (Fig. 1A and 2). This result is unlike previous studies where *C. ragsdalei* could not grow in yeast free medium [22]. However, the productivity observed in the current study was a bit lower [23–25]. The results indicate the importance of the yeast to the productivity of *C. ragsdalei*. Gao et al. [25] observed a significant lower acetate and ethanol concentration in a yeast free medium compared to a yeast medium. It could be due to that the components in yeast such as carbohydrates and vitamins could contribute to better growth of *C. ragsdalei* and thus lead to a higher concentration of products.

Propionate was the second most dominant product during fermentation with *C. ragsdalei*. It was produced in all compositions in concentrations higher than 2 mmol/l after 4 days. The maximum concentration of propionate was observed under a headspace of CO with a value of 3.1 mmol/l. Only few pathways for propionate production have been reported for *Clostridium* species. Coma et al. [18] suggested that

propionate could be formed by chain elongation of formate with ethanol as reductant (Table 2, reaction 8). Formate has been found to be one of the products by microbial fermentation of syngas [26]. Formate and ethanol were assumed to be produced simultaneously and used as precursors for propionate formation. According to Eq. (1) and (2) the formation of propionate (reaction 8) should become more favorable with ethanol being formed simultaneously. Another pathway for propionate production is through the acrylate pathway however this has only been described for very few bacterial species [5].

Maximum production of butyrate and valerate were also observed under a headspace of CO with a concentration of 1.2 and 0.8 mmol/l, respectively. The final concentration of butyrate and valerate under the headspace of SS was 29% and 40% lower than that under CO. This again suggests that oxidizing enzymes might have been inhibited by the presence of both CO and H₂. Overall a headspace of CO showed to be the most favorable gas composition with respect to final concentrations of VFAs. The concentration of TVFAs produced under CO was 35% higher than that under headspaces of SS. Fermentation with *C. ragsdalei* showed negligible formation of products under the control (Fig. 2C), which indicated the importance of having either CO or H₂ as reductant for product formation to occur.

The production of ethanol and VFAs using mixed culture was also investigated. The most dominant product in both gas compositions was acetate, while no ethanol was produced under the headspace of CO and SS (Fig. 3). The maximum acetate concentration was achieved under a headspace of CO which was 19.4% higher than under headspaces of SS. This clearly indicated that CO was preferred over mixture of CO and H₂ (as syngas) for acetate production by the mixed culture. The maximum

acetate concentration reported by Singla et al. [11] through a syngas fermentation with TERI SA1 anaerobic mixed cultured was five and four-fold that produced under SS and CO headspace in the current study. Besides, the absence of yeast on the fermentation medium and the heating treatment of the inoculum could be another possible reason for the low productivity of the mixed culture. Wang and Yin [27] highlighted the effect of temperature on the microbial survival. Meanwhile, several adverse effects of. No significant difference ($p > 0.05$) in final concentrations of other VFAs was observed during fermentation under a headspace of SS and CO. These results were also confirmed by other researchers who reported the ability of acetogens to grow chemolithotrophically on CO alone or syngas and ferment them to acetate as a main microbial end product [5,11]. Overall mixed culture clearly showed to prefer headspaces of CO with respect to final concentrations of VFAs.

3.3. The effect of acetate on the gases fermentation

Fermentation by *C. ragsdalei* in all three gas compositions with acetate modified medium (Fig. 4A, B, C) showed that acetate was the most dominant product. The maximum concentration of acetate was observed under the headspace of CO which was 75% and 127.2% higher than that achieved under SS and H₂ headspace. The maximum concentration of acetate achieved under the headspace of CO, was 22% less compared to fermentation without addition of acetate. This suggested a negative effect on acetate formation when it already present in the medium. The negative effect could be explained by a change in thermodynamic conditions when acetate was added initially. From Eq. (1) and (2) it can be seen that when the initial concentration of a product (in this case acetate) is high, the ΔG for the reaction will decrease. Fig. 4A and B showed that the addition of acetate during pure culture fermentation under headspaces of SS and

CO resulted in a significant increase ($p < 0.05$) of butyrate formation. The maximum concentration of butyrate (2.52 mmol/l) was observed under the headspace of CO, which was 29.2% and 240.5 higher than that produced under SS and H₂ headspace. The highest concentration of butyrate measured under a headspace of CO and SS was 115% and 134% significantly higher ($p < 0.05$) compared to the same experiment without addition of acetate. This increase in butyrate concentrations with *C. ragsdalei* could be explained by that chain elongation reactions were becoming more favorable as a result of initial presence of acetate. It has not been so far documented that *C. ragsdalei* have the ability to perform reverse β -oxidation for butyrate formation. Results from this study however indicate that this could be a possibility. According to calculations of the actual Gibbs free energy change (ΔG) at real fermentation conditions (Eq. (1) and (2)), high acetate concentrations would result in increased ΔG for formation of acetate from syngas, (reaction 5 in Table 2), while the ΔG would decrease for reduction of acetate to ethanol with CO as reductant (in reaction 15). Consequently the formation of butyrate from ethanol and acetate (reaction 10) would also become more favorable potentially explaining the increase in formation. Alternatively the increased concentration of butyrate as a result of adding acetate, could be due to that the formation of acetate directly from CO became less favorable (reaction 5), resulting in direct butyrate formation from CO (reaction 11) occurring instead. An increase in butyrate concentration of more than 100% was considered a significant improvement. Two other studies however reported concentrations of butyrate to be 229% [8] and 287% [2] higher than the maximum concentration obtained in this study. One reason could be that the concentration of buffer solution was significantly higher in this study. Phillips et al. [2] used no MES buffer, while Ramachandriya et al. [8] employed less than 30% of the

concentration used in the present study. MES buffer has been reported to have a negative effect on product formation [25,28] and the high concentration used in this study was therefore considered likely to have inhibited further increase of butyrate production. However more valuable products (butyrate and valerate) increased as a result of acetate addition but the overall concentration of VFAs was lower compared to experiment without acetate addition. Under headspaces of CO and SS, the decrease in all measured VFAs was 11% and 19%, respectively compared to when no acetate was added. When the headspace was filled with SS, the addition of acetate increased the rate of formation of ethanol compared to that without acetate addition. The maximum concentration of ethanol (2.03 mmol/l) produced by *C. ragsdalei* was observed under a headspace of SS after day 4 of inoculation, which was 42% and 151% higher than ethanol produced under CO and H₂, respectively. Final concentrations of ethanol in headspaces of SS and CO however did not clearly change as a result of acetate addition. This could be due to that ethanol was directly used as reductant during chain elongation as well as an increase in pH (Supplementary Information (SI)). The addition of acetate during fermentation resulted in a positive effect on ethanol formation under all the gases conditions (Fig. 4C). According to Eq. (1) and (2) the reduction of acetate to ethanol (reaction 16 in Table 2) would also become more favorable with a higher initial concentration of acetate.

The effect of acetate addition on the gas fermentation was also investigated with mixed culture. Acetate was the main metabolite produced from mixed culture fermentation (Fig. 5). The maximum concentration of acetate by mixed culture fermentation was observed under a headspace of CO. It was further observed that under a headspace of CO the presence of acetate enhanced its own formation by 6% compared

to experiment without acetate addition. The addition of acetate showed negative effect under headspaces of SS, where the formation of acetate was 42% lower than under headspace of SS. Due to acetate being the most dominant product, the same pattern was observed for overall production of VFAs. Under a headspace of CO, end product formation increased by 7% as a result of adding acetate, while it decreased by 34% under headspaces of SS. The formation of propionate, butyrate and valerate with mixed culture was slightly higher with acetate addition (Fig. 5). Under headspaces of SS and CO, butyrate increased by 19% and 7% respectively, compared to the experiments without acetate addition. The addition of acetate had no effect on ethanol under any of the gas compositions during mixed culture fermentation. This was assumed to be due to increasing pH throughout inoculation (Supplementary Information (SI)). According to previous studies [29,30] increasing pH inhibits formation of alcohol while chain elongation is enhanced. Besides, Jankowska et al. [31] observed higher production of fatty acids at high pH (with maximum concentrations around pH 10-11). Furthermore, it was observed that longer inoculation times (10-15 days) the higher concentrations were achieved. Thus, a high initial pH could therefore potentially have enhanced the production of fatty acids in this study.

4. Conclusions

Through this study, acetate was the most dominant product independent of inoculum type. With respect to end product concentrations, pure culture of *C. ragsdalei* showed to be superior to the mixed culture. Depending on the headspace gas composition, VFAs concentrations were up to 300% higher after fermentation with pure culture compared to mixed culture. The preferred gas composition with respect to highest concentrations of

VFAs was CO regardless of microbial composition. In pure culture fermentation, a headspace of CO resulted in up to 35% higher VFAs than that produced under the headspace of SS. While, in the mixed culture resulted in up to 18% higher VFAs compared to SS headspace. The maximum concentration of ethanol was also achieved under the headspace of CO by *C. ragsdalei*. while no ethanol was produced by mixed culture fermentation in any of the both gas compositions. Addition of acetate through the pure culture fermentation resulted in higher concentrations of butyrate and valerate compared to the experiment without acetate addition. However, the overall concentration of VFAs was lower compared to experiment without acetate addition. Under headspaces of CO and SS, the decrease in all measured VFAs was 11% and 19%, respectively compared to that without acetate addition. The effect of added acetate in mixed culture fermentation varied depending on the headspace composition. Results obtained from this study can pave the way towards sustainable valorization of waste CO/CO₂ gases for biofuels and biochemicals production.

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Figure Captions

Fig. 1. Growth profile (optical density, OD₆₀₀) of: A) pure and B) mixed culture with acetate free media.

Fig. 2. Ethanol and VFAs production profile of the pure culture with acetate free media observed under different gas composition: (A) Synthetic syngas, (B) pure CO and (C) control gas consisting of only N₂.

Fig. 3. Ethanol and VFAs production profile of the mixed culture with acetate free media observed under different gas composition composition: (A) Synthetic syngas, (B) pure CO and (C) control gas consisting of only N₂.

Fig. 4. Ethanol and VFAs production profile of the pure culture with acetate modified media observed under different gas composition: (A) Synthetic syngas, (B) pure CO, (C) pure H₂ and (D) control gas consisting of only N₂.

Fig. 5. Ethanol and VFAs production profile of the mixed culture with acetate modified media observed under different gas composition: (A) Synthetic syngas, (B) pure CO, (C) pure H₂ and (D) control gas consisting of only N₂.

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501	Tables
502	Table 1 Experimental design for effect of media, feed gas and microbial composition
503	during the fermentation process.
504	Table 2 ΔG° of possible fermentation reactions by Clostridium and mixed cultures
505	under standard condition.

Table 1

Table 1

Treatment		Factors	
Levels	Media	Culture	Gas composition
0	AFM	Pure culture	Syngas (60%CO: 35% H ₂ :5%CO ₂)
1	AMM	Mixed culture	Pure CO (100%)
2			Pure H ₂ (100%)
1	0	0	0
2	0	1	0
3	0	0	1
4	0	1	1
5	1	0	0
6	1	1	0
7	1	0	1
8	1	1	1
9	1	0	2
10	1	1	2

Table 2

Chain Elongation (incl. Wood-Ljungdal pathway)				
Stoichiometry	#	ΔG° * (kJ/mol)	Reference	
Ethanol	$6CO + 3H_2O \rightarrow CH_3CH_2OH + 4CO_2$	1	-217.4	[1]
	$3CO + 3H_2 \rightarrow CH_3CH_2OH + CO_2$	2	-157.2	[1]
	$2CO + 4H_2 \rightarrow CH_3CH_2OH + H_2O$	3	-137.1	[1]
	$2CO_2 + 6H_2 \rightarrow CH_3CH_2OH + 3H_2O$	4	-97.0	[2]
Acetate	$4CO + 2H_2O \rightarrow CH_3COO^- + H^+ + 2CO_2$	5	-154.6	[1]
	$2CO + 2H_2 \rightarrow CH_3COO^- + H^+$	6	-114.5	[1]
	$2CO_2 + 4H_2 \rightarrow CH_3COO^- + H^+ + 2H_2O$	7	-54.8	[3]
Propionate	$HCOO^- + CH_3CH_2OH \rightarrow CH_3CH_2COO^- + H_2O$	8	-65.4	[3]
Butyrate	$2CH_3COO^- + H^+ + 2H_2 \rightarrow CH_3(CH_2)_2COO^- + 2H_2O$	9	-88.0	[3]
	$CH_3COO^- + CH_3CH_2OH \rightarrow CH_3(CH_2)_2COO^- + H_2O$	10	-38.5	[3]
	$10CO + 4H_2O \rightarrow CH_3(CH_2)_2COO^- + H^+ + 6CO_2$	11	-420.8	[2]
	$10H_2 + 4CO_2 \rightarrow CH_3(CH_2)_2COO^- + H^+ + 6H_2O$	12	-220.2	[2]
Valerate	$2CH_3CH_2COO^- + H^+ + 2CO_2 + 6H_2 \rightarrow CH_3(CH_2)_3COO^- + H_2O$	13	-142.8	[3]
	$2CH_3CH_2COO^- + H^+ + CH_3COO^- + H^+ \rightarrow CH_3(CH_2)_3COO^- + 2H_2O$	14	-88.0	[3]
Reduction of acetate to ethanol				
Ethanol	$CH_3COO^- + H^+ + 2CO \rightarrow CH_3CH_2OH + 2CO_2$	15	-62.5	[4]
	$CH_3COO^- + H^+ + 2H_2 \rightarrow CH_3CH_2OH + H_2O$	16	-22.3	[5]

* ΔG values were calculated using enthalpy and entropy values under standard condition i.e. 0°C, 1 atm, 1 molar or 1 atm partial pressure liquid and gaseous concentration respectively.

Figure 1

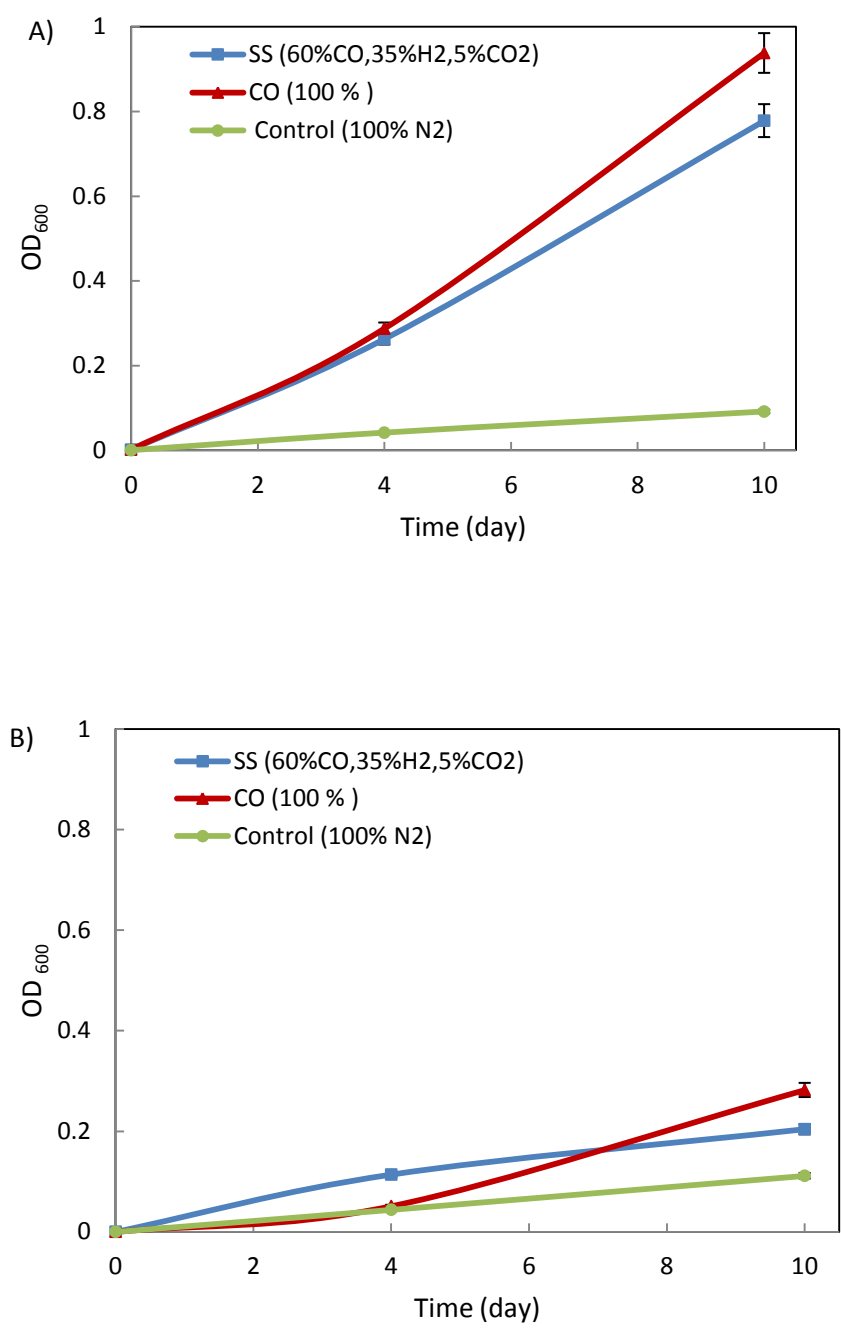


Fig. 1.

Figure 2

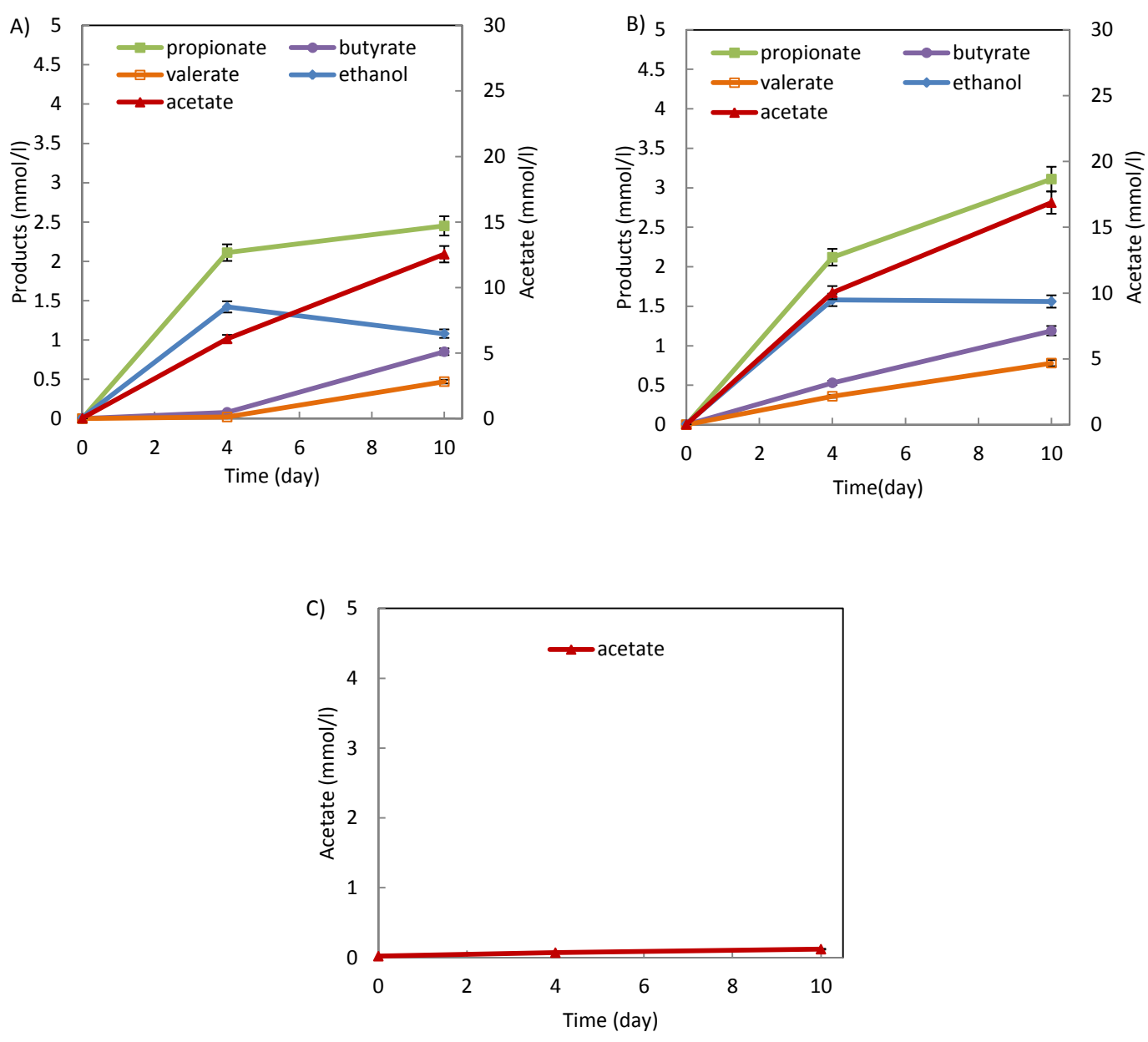


Fig. 2.

Figure 3

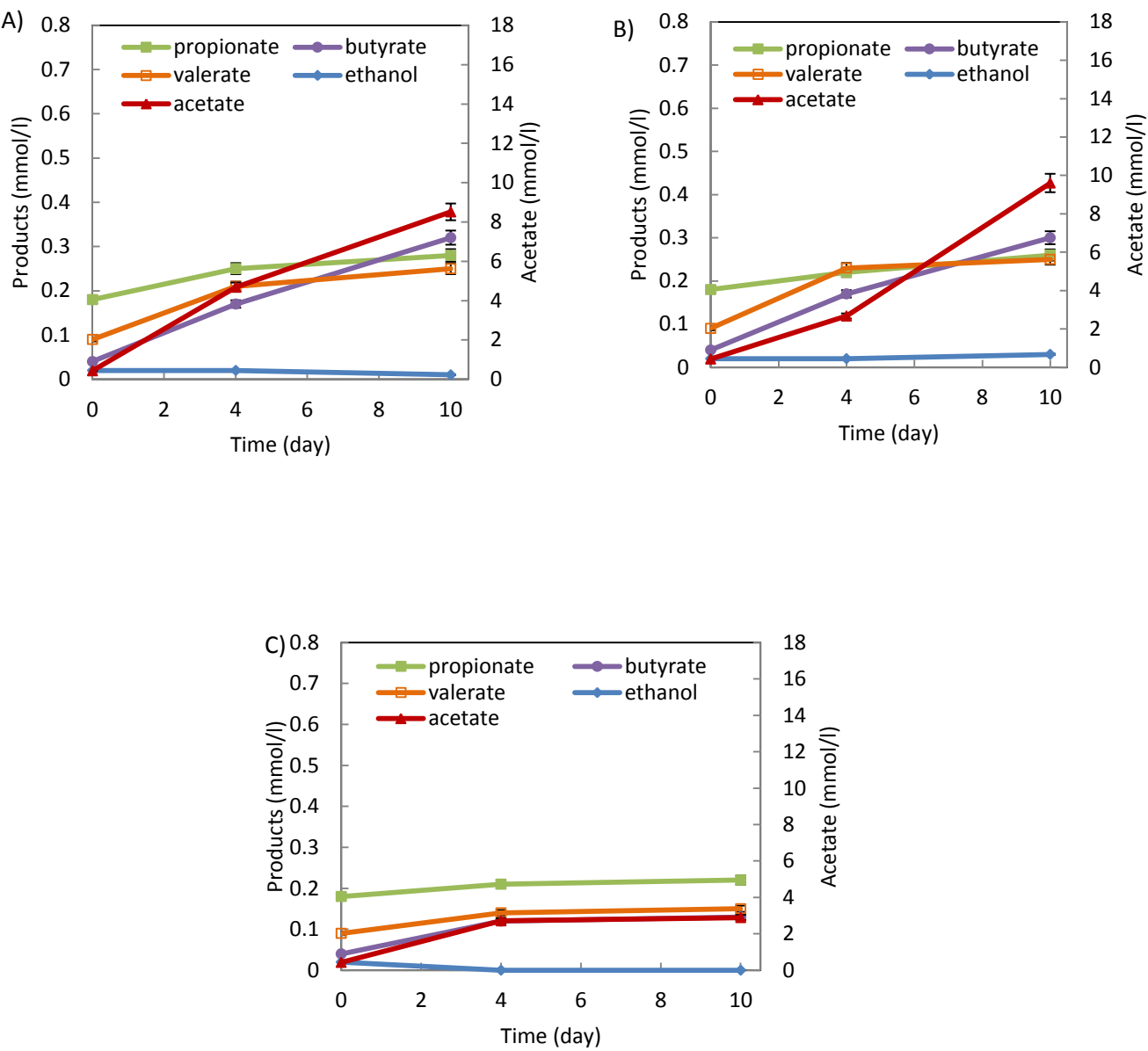


Fig. 3.

Figure 4

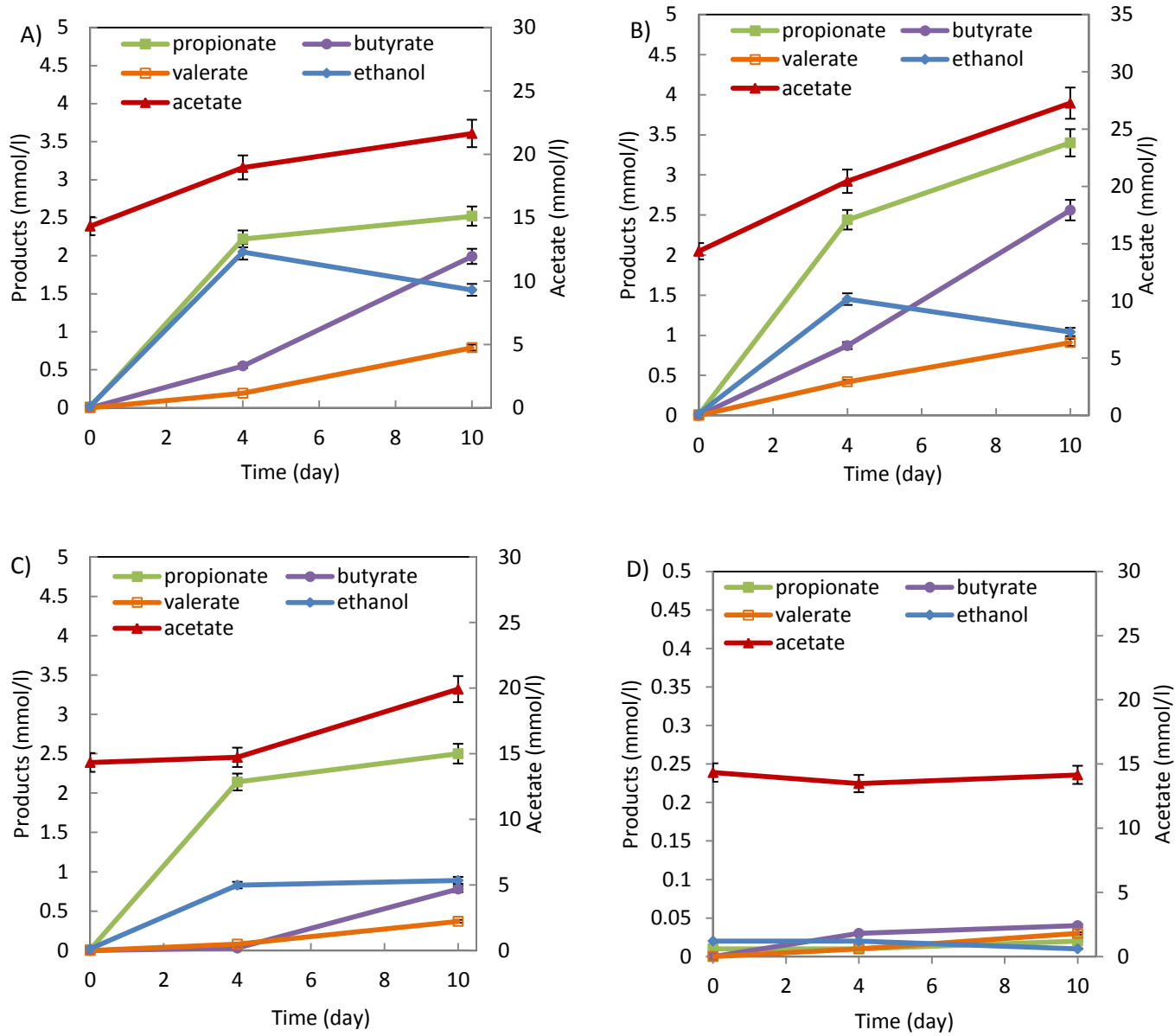


Fig. 4.

Figure 5

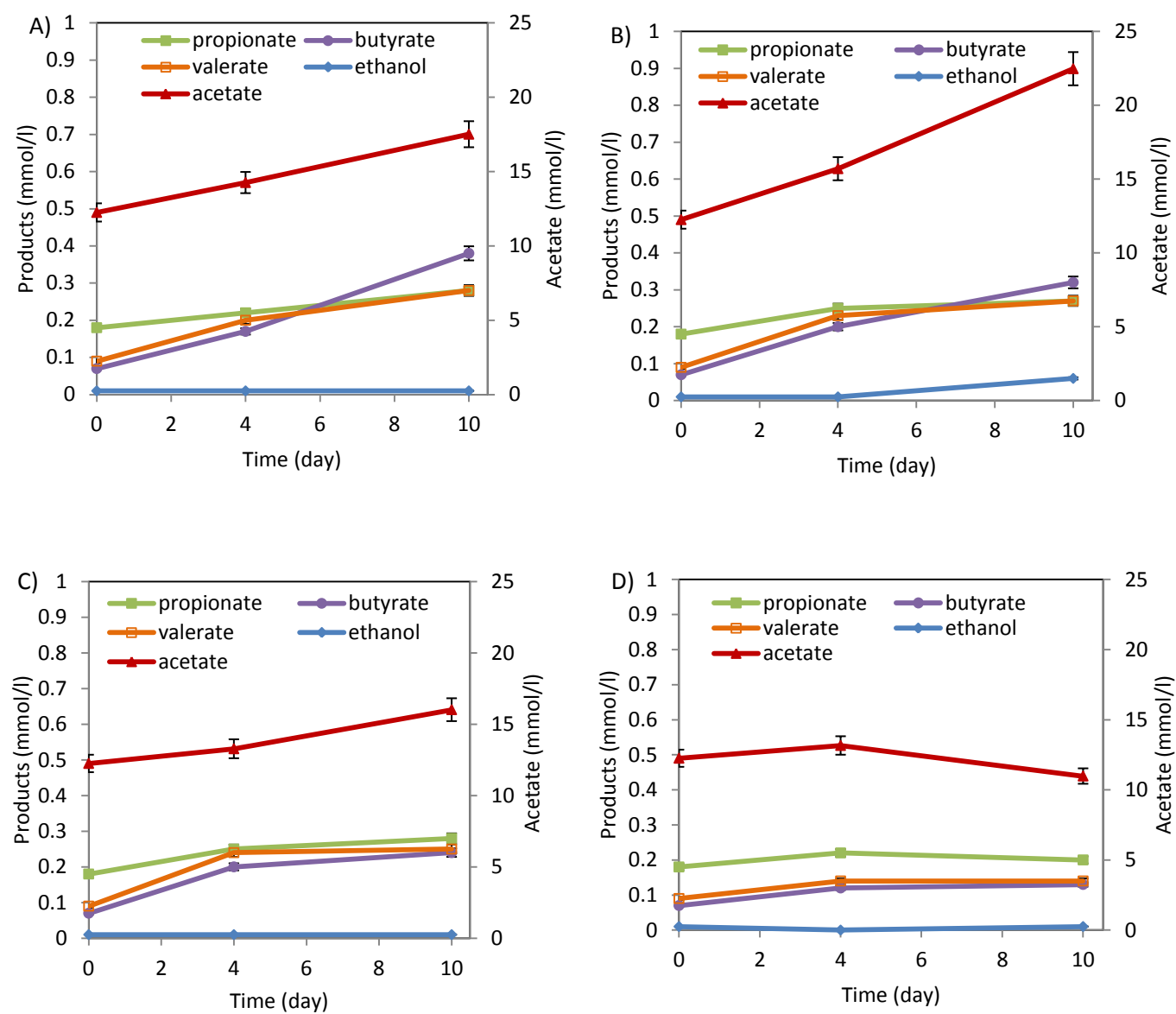


Fig. 5.

High efficient ethanol and VFAs production from gas fermentation: effect of acetate, gas compositions and microbial culture

Maie El-Gammal ^a, Reda Abou-Shanab ^b, Irini Angelidaki ^c, Basma Omar ^{a,c}, Per Viktor Sveding ^c, Dimitar Borisov Karakashev ^c, Yifeng Zhang ^{c,*}

Supplementary information includes:

Pages 1 to 2

Materials and Methods

Fermentation Media

Detailed media composition of vitamin; trace metal and reducing agent stock solutions used in the Clostridium strain P11 standard media formulation (Phillips et al., 2015).

Trace element stock solution	g/l
Nitrilotriacetic acid	2
MnSO ₄ •H ₂ O	1.0
Fe(SO ₄) ₂ (NH ₄) ₂ •6H ₂ O	0.8
CoCl ₂ •6H ₂ O	0.2
ZnSO ₄ •7H ₂ O	0.2
CuCl ₂ •2H ₂ O	0.02
NiCl ₂ •6H ₂ O	0.02
Na ₂ MoO ₄ •2H ₂ O	0.02
Na ₂ SeO ₄	0.02
Na ₂ WO ₄	0.02
Vitamin stock solution	
Biotin, vitamin B ₇	0.02
Folic acid	0.02
Pyridoxine HCl, vitamin B ₆	0.1
Riboflavin, vitamin B ₂	0.05
Thiamine HCl, vitamin B ₁	0.05
Cyanocobalamine, vitamin B ₁₂	0.001
Nicotinic acid	0.05
P-Aminobenzoic acid, PABA	0.05
Lipoic acid	0.05
DL-panthothenic acid	0.05
Reducing agents stock solution	
NaOH	9
L-Cysteine•HCl	40
Na ₂ S•9H ₂ O	40

pH profile during the fermentation process

Biocatalyst	Media	Gas composition	Initial pH	Final pH
<i>C. ragsdalei</i>	AFM	SS	5.9	6.7
		CO	5.9	6.6
	AMM	SS	5.9	6.4
		CO	5.8	6.2
		H ₂	6.1	6.7
Mixed culture	AFM	SS	6.0	7.0
		CO	5.9	7.1
	AMM	SS	5.8	7.4
		CO	5.9	7.0
		H ₂	5.9	8.6

AFM, acetate free media – AMM, acetate modified media

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J.R. Phillips, H.K. Atiyeh, R.S. Tanner, J.R. Torres, J. Saxena, M.R. Wilkins, R.L. Huhnke, Butanol and hexanol production in *Clostridium carboxidivorans* syngas fermentation: Medium development and culture techniques, *Bioresour. Technol.* 190 (2015) 114–121. doi:10.1016/j.biortech.2015.04.043.